09/930,329

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/83, C12Q 1/68, A01H 5/00, A01K 67/027

A1

(11) International Publication Number: WO 91/13994

(43) International Publication Date: 19 September 1991 (19.09.91)

(21) International Application Number: PCT/AU91/00088

(22) International Filing Date: 13 March 1991 (13.03.91)

(30) Priority data:
PJ 9093 13 March 1990 (13.03.90) AU

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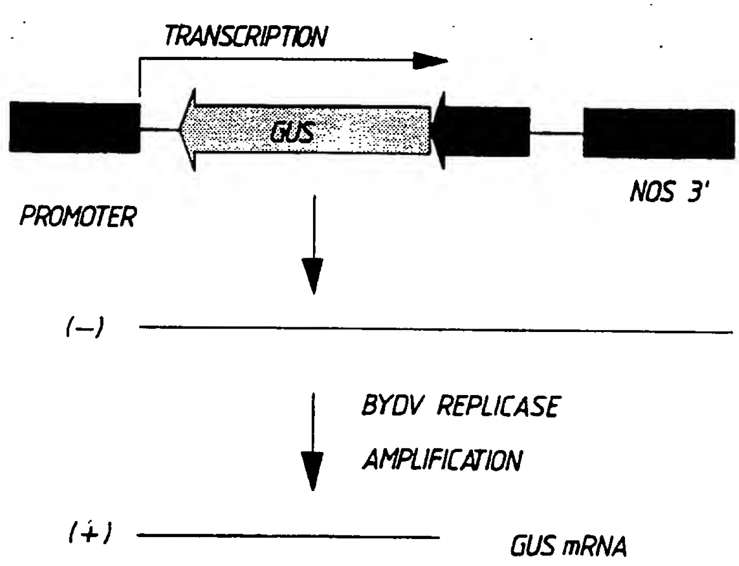
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: GENE EXPRESSION



(57) Abstract

A nucleic acid sequence comprising: (a) a transcriptional promoter; (b) a heterologous gene sequence operably linked to said transcriptional promoter; and (c) a sub-genomic promoter ligated to said heterologous gene sequence; such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript. There is also described eukaryotic cells, and transgenic plants and animals containing said nucleic acid sequences, which may be phenotypically altered and/or protected from viral infection by one or more products of the heterologous gene sequence, or which may be capable of producing polypeptide products encoded by the heterologous gene sequence.

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GENE EXPRESSION

This invention broadly relates to gene expression and in particular relates to nucleic acid sequences capable of providing amplified levels of heterologous gene transcripts both in-vivo and in-vitro. This invention further relates to host cells, plants and animals containing said nucleic acid sequences.

There have been many proposals for transcriptional amplification, most centering on the use of a strong promoter to drive efficient and sustained transcription.

Transcribed mRNA of a desired gene or genes may be translated into desirable polypeptide products, useful, for example, as therapeutics, biological reagents, antibiotics, antivirals and the like.

In plants, particularly transgenic plants, RNA transcripts of a desired gene may encode polypeptides which modify phenotype, for example, by affecting sterility, salt tolerance, viral susceptibility, drought tolerance, acidity, colour and the like.

In animals, particularly transgenic animals, RNA transcripts of a desired gene or genes may be translated into polypeptide products having a host of phenotypic actions/effects, these being dependent upon the nature of the polypeptide product.

RNA transcripts may have activity in their own right, such as antisense reagents which block translation or inhibit RNA function, or as endoribonucleases (sometimes known as ribozymes) which can effect catalytic cleavage of target RNA molecules.

Previous approaches for transcriptional elevation have, at least to some extent, been hampered by low levels of transcription, which may in part be due to promoters having poor activity, or other less defined causes.

In accordance with the first aspect of this invention, there is provided a nucleic acid sequence characterised in that it comprises:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript.

The nucleic acid sequence of this invention is generally in the form of DNA or derivatives thereof, and may be single or double-stranded. DNA "derivatives" are well known in the art and include base modifications, modifications of the sugar moiety, modifications of the phosphodiester linking groups, and modifications of the 5' and 3' termini. The oligonucleotide backbone (that is the phosphodiester linkages) may be modified in a variety

of ways, for example, in the same manner as for DNA antisense oligonucleotides. Methylphosphonate or phosphorothioate linkages may be used to replace conventional phosphodiester linkages. Nucleotide bases and sugar moieties may be substituted with one or more substituents such as halogen, alkyl, alkoxy, and the like. Carbon atoms, nitrogen atoms or oxygen atoms forming part of the nucleotide base or sugar moiety may be replaced with different atoms such as carbon, sulphur, nitrogen, halogen and the like. Deoxynucleotides may be replaced with ribonucleotides. As previously mentioned, the above modifications are well known in the art, and are described, for example in Buck et al. (Science 248: 208-212 (1990)).

Where nucleic acid sequences are double-stranded, the complementary strand is selected to have complementary sequence.

Nucleic acid sequences may additionally comprise a selectable marker, such as an antibiotic or herbicide resistance gene, and may further include nucleotide sequences to facilitate integration of said nucleic acid sequences into chromosomal DNA of eukaryotic or prokaryotic cells, or poly adenylation sequences. For example, where integration of nucleic acid sequences into plant cells is desired, the nucleic acid sequences of this invention may contain T-DNA of the Ti-plasmid of Agrobacterium. Of course, other types of nucleotide sequences can be used to facilitate integration into the genome of eukaryotic or prokaryotic cells. In respect of eukaryotic cells, viral sequences such as long terminal repeats (LTR's) may be employed.

Where a protein product is to be produced utilizing the nucleotide sequence of this invention, the heterologous gene sequence is in an orientation (inverted) which gives rise to a negative sense RNA on transcription (primary transcript), also known as a minus (-) strand, whose nucleotide sequence corresponds to the

non-coding strand of a heterologous gene sequence. This minus strand is not capable of being translated to the desired protein product, as it is in the wrong orientation and would give a nonsensical product if translated. However, transcription driven from the sub-genomic promoter located on the primary transcript at the 3' end thereof produces a positive sense RNA transcript which may then be translated to give a desired protein product.

The opposite situation exists where the secondary RNA transcript is desired to be an anti-sense RNA or a ribozyme. The primary RNA transcript is in a positive (+) sense. Transcription from the sub-genomic sequence produces a negative (-) sense RNA (secondary transcript) which may then act as an anti-sense RNA or an endoribonuclease.

Nucleotide sequences of this invention may comprise all or part of a transfer vector such as a plasmid or phage transfer vector. Transfer vectors may contain nucleotide sequences encoding selectable markers, such as antibiotic resistance and may include nucleotide sequences to facilitate integration of said nucleic acid sequences into chromosomal DNA of eukaryotic or prokaryotic cells.

Plasmids containing the nucleic acid sequences of this invention would comprise an origin of replication to enable plasmid replication inside host cells.

Transcriptional promoter sequences may be selected from any nucleotide sequences which bind or interact with DNA dependent RNA polymerase to effect transcription of DNA sequences operably linked to said promoter. Preferably said promoters are of eukaryotic origin, and in a preferred embodiment of this invention, are of plant origin and may, for example, be plant viral promoters. Suitable promoters are well known in the art and are described for example in O'Neal et al. (Nucleic Acids. Res. 15: 8661 (1988)) and Harpster et al. (Mol. Gen.

Genet. 212: 189-190 (1988)).

Transcriptional promoters may be induceable by various stimuli, such as temperature, salinity, moisture, stress, viral infection and the like.

Transcriptional promoter sequences may contain more than one promoter, to enable transcriptional induction under various stimuli or conditions.

The heterologous gene sequence may encode any desired peptide product, such as hormones (e.g. insulin, relaxin, growth hormone and LHRH), toxins, antibiotics, such as proteins which interfere with virus infection, biological reagents, proteases which degrade cellular proteins, or two or more such peptide products. In respect of plants, said heterologous nucleic acid sequences may encode polypeptides which modify phenotype, sterility, salt tolerance, fruit ripening viral susceptibility, drought tolerance, acidity, colour, and the like, or proteases which degrade cellular proteins. It is important to appreciate that the precise nature of the polypeptide product(s) encoded by a heterologous nucleotide sequence is not of importance and may be selected according to a particular desired purpose.

In a preferred aspect of this invention, the protein product encoded by a heterologous gene is a protein which disrupts viral replication in plant or animal cells. In such circumstances, the nucleic acid sequence of this invention is generally integrated into the chromosomal DNA of a plant or animal cell by methods well known in the art.

As hereinbefore mentioned, where a protein product is to be produced utilizing the nucleic acid sequence of this invention, the heterologous gene sequence encoding the protein product is present in an inverted form to produce a primary transcript corresponding to the non-coding strand of the heterologous gene sequence.

The heterologous gene sequence may encode RNA transcripts which function as antisense RNAs, that is,

are of complementary sequence to specific cellular RNAs in eukaryotic or prokaryotic cells, which may, for example, be concerned with the translation of protein products, viral replication, cell division, metabolism, differentiation, division, sterility, phenotypic traits or a host of cellular processes. Under such circumstances, the nucleotide sequence of the heterologous gene sequence is, of course, selected to be substantially complementary thereto, and therefore capable of hybridization thereto, thus inactivating a desired exogenous/endogenous/pathogenic RNA sequence within a cell.

Heterologous nucleotide sequences may encode one or more endoribonucleases, such as described, for example, by Haseloff and Gerlach (Nature 334: 585-591 (1988)) and Cech et al. (U.S. Patent No. 4,987,071). Such endoribonucleases are capable of site specific cleavage of target RNAs, and therefore may be used to inactivate any desired cellular RNA, which may, for example, be involved in viral replication, or cellular regulation or processes (such as division, differentiation, and the like).

As referred to herein, the heterologous gene sequence encoding an anti-sense RNA or endoribonuclease directs transcription of a positive sense (+) transcript, which on transcription from the sub-genomic promoter produces a negative sense (-) secondary transcript.

The sub-genomic promoter is operably linked to the heterologous gene sequence such that on transcription of the nucleic acid sequence to give a primary transcript, the sub-genomic promoter then drives transcription of the heterologous nucleic acid sequence to produce a secondary amplified transcript. The secondary transcript is capable of translation to give a polypeptide encoded by said heterologous nucleic acid sequence.

The sub-genomic promoter binds or interacts with RNA dependent RNA polymerase, and thus is generally of viral

origin, particularly plant or animal viral origin.

A number of positive stranded RNA viruses use a strategy of sub-genomic RNAs to amplify and express internal cistrons in their genomic RNA. These are transcribed from the negative strand replicating form of the virus using signals known as "sub-genomic promoters" in the RNA template to commence transcription at the defined regions. This strategy provides for production of translatable RNAs from these internal genes. Sub-genomic promoters have been described for a range of plus stranded plant and animal viruses (see French and Ahlquist, J. Virol. 62: 2411-2420 (1988); Levis et al., J. Virol. 64: 1726-1733 (1990) and scientific papers referred to therein).

The nature of the sub-genomic promoter will, of course, depend upon the particular circumstances in which the nucleic acid sequence of this aspect of the invention is employed. For example, when the nucleic acid sequence is integrated into a plant genome or carried as an extra chromosomal element within plant cells, the sub-genomic promoter may be selected to correspond to a sub-genomic promoter of an infecting plant virus. The viral RNA polymerase of the particular virus mediates transcription from the sub-genomic promoter of the heterologous nucleotide sequence which may encode a polypeptide, endoribonuclease, or anti-sense RNA to inactivate the infecting virus. A similar approach may be adopted in animal cells, where the sub-genomic promoter may be selected to be specific for the RNA polymerase of an infecting virus. In both of these situations, viral infection would trigger transcription, which in turn would lead to viral modulation/inactivation.

The sub-genomic promoter may comprise multiple promoter sequences wherein each sub-genomic promoter sequence of the multiple sub-genomic promoter sequences is recognised by a specific RNA dependent RNA polymerase. For example, in plants or animals, promoters

corresponding to various plant or animal viruses may be utilised. As RNA polymerases are generally promoter specific, the use of multiple promoters enables transcription of the heterologous gene sequence on viral infection with a number of viral types. In such circumstances, the heterologous nucleotide sequence may encode a cytotoxic product which causes cell death or which inhibits viral function, such as by the inhibition of reverse transcriptase, disruption of viral particle assembly, or modulation or replication.

The nucleic acid sequence of this invention may additionally comprise a complementary sequence of a sub-genomic promoter inserted between the transcriptional promoter and the heterologous gene sequence, such that the secondary transcripts are transcribed from the sub-genomic promoter produced on transcription of the primary transcript (from the complementary sequence of the sub-genomic promoter) to give a plurality of tertiary transcripts.

In this embodiment the heterologous gene sequence encoding a desired polypeptide would be in an orientation selected to give rise to positive sense (+) RNA. Two rounds of amplification provided by the sub-genomic promoters would produce an amplified positive sense (+) tertiary transcript which may be translated to give a desired polypeptide product.

In a second aspect of this invention, there is provided a eukaryotic cell which contains a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript,

and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript.

The nucleic acid sequence as hereinbefore described may be integrated into the genome of the eukaryotic cell or may be present as an extra chromosomal element, such as a plasmid.

Transfection of eukaryotic cells is carried out according to well known methods in the art, for example by using plant or animal viruses which integrate into chromosomal DNA, or plasmids such as the Ti-plasmid of Agrobacterium, pollen mediated transformation, in-vitro protoplast transformation, lyposome-mediated transformation, and other well known methods such as are described in .

Preferably, the eukaryotic cell is a plant or animal cell.

In a further aspect of this invention, there is provided a transgenic plant containing a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript.

The transgenic plant may be wheat, barley, rye, sorghum, potato, rice, rape, apple, and the like.

In a still further aspect of this invention, there is provided a transgenic animal containing a nucleic acid sequence:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript.

Plant and animal transgenes may contain the nucleic acid sequence of the first aspect of this invention described hereinbefore in some or all of the cells thereof, integrated into the genome and/or carried on extra chromosomal elements.

In the various embodiments of the invention where cells, plants or animals contain the aforementioned nucleotide sequence either integrated into the genome or carried as an extra-chromosomal element, said cells, plants or animals may contain additional nucleotide sequences which encode an RNA dependent RNA polymerase which is capable of effecting transcription from the sub-genomic promoter under control of one or more promoter sequences. These promoter sequences may be induced by the same stimuli which induces transcription from the transcriptional promoter. Under these circumstances transcribed primary transcripts RNA encoding one or more heterologous gene products would be rapidly transcribed by constitutively produced polymerase to give a secondary transcripts or tertiary transcripts which may then be translated to give a protein product.

The gene encoding the RNA dependent polymerase under promoter control may be integrated into the genome or be carried as an extra-chromosomal element. It may be provided as a construct with the nucleic acid sequence of this invention, where the nucleic acid sequence and the polymerase gene plus promoter are separated by

transcriptional termination sequences, such that the nucleic acid sequence and the polymerase gene are separately transcribed.

In a yet further aspect of this invention, there is provided a process for transcriptional amplification, which process comprises:

(i) providing a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

(ii) reacting said nucleic acid sequence with a DNA dependent RNA polymerase such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript; and

(iii) reacting said primary transcript with an RNA dependent RNA polymerase to thereby effect transcription of said primary transcript from the sub-genomic promoter to give a plurality of amplified secondary transcripts.

The above method may be carried out in-vivo or in-vitro.

In still a further aspect of this invention, there is provided a method for protecting a plant or animal cell from viral infection, which method comprises introducing into one or more cells of said plant or animal a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription

under the action of said sub-genomic promoter to give a secondary transcript;

and wherein the primary transcript produced under control of said transcriptional promoter is transcribed from the sub-genomic promoter by an RNA polymerase to give a secondary transcript which encodes a polypeptide product, or which itself, inhibits viral replication or inactivates said virus.

In another aspect of this invention there is provided a method of producing a heterologous gene product in a cell, which method comprises introducing into said cell a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter;

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript; and

reacting said primary transcript produced on cellular transcription of said nucleic acid sequence with an RNA dependent RNA polymerase capable of transcription from said sub-genomic promoter to give a secondary transcript which itself may comprise said heterologous gene product, or which may be translated in the cell to give a heterologous gene product.

In accordance with another method of this invention there is provided a method for inactivating an exogenous or endogenous cellular RNA, which method comprises introducing into a cell a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to

said transcriptional promoter;

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript;

wherein the primary transcript produced under control of said transcriptional promoter is transcribed from the sub-genomic promoter by an RNA polymerase to give a secondary transcript, and wherein said secondary transcript or a translated product thereof inactivates said exogenous or endogenous cellular RNA.

Exogenous RNA's may be any foreign RNA such as a viral RNA or RNA of a pathogenic organism. An endogenous RNA is any cellular RNA.

In the above methods cells may be transfected with an RNA dependent polymerase under promotional control, such that the polymerase may be produced within the cells under appropriate stimuli which activate said promoter.

Alternatively, on infection with a virus whose RNA polymerase recognises the sub-genomic promoter, transcription is effected to give a positive sense RNA, which itself, or its translated product, is effective to inhibit said virus.

In the aforementioned methods of this invention where a complementary sequence of a sub-genomic promoter is inserted between the transcriptional promoter and the heterologous gene sequence, the amplified tertiary transcripts or encoded polypeptide products may act to inhibit/inactivate virus, inactivate exogenous or endogenous cellular RNA, modify cellular phenotype, and the like.

The nucleic acid sequence of this invention amplifies transcription by employing at least two

promoters. The first promoter (transcriptional promoter) enables production of a primary transcript. Each primary transcript is then in turn transcribed using the secondary promoter located on the primary transcript (sub-genomic promoter) to give a secondary transcript which is capable of translation.

For each primary transcript, many secondary transcripts may be produced resulting in significant transcriptional amplification.

Where a complementary sequence of a sub-genomic promoter is inserted between the transcriptional promoter and the heterologous gene sequence further transcriptional amplification is enabled. Particularly, the active sequence of the sub-genomic promoter (derived from the complementary sequence thereof) is provided in the secondary transcript. Transcription is then driven from the amplified secondary transcript by the sub-genomic promoter present thereon.

It will be apparent that heterologous gene transcripts may thus be amplified considerably, when compared to the situation in which the heterologous gene is oriented in the positive orientation, and transcription thereof is singly driven by the transcriptional promoter.

The sub-genomic promoter is generally recognised, as mentioned above, by a viral RNA dependent RNA polymerase (viral replicase complex) thus providing applications in-vivo (that is in eukaryotic and prokaryotic cells) as follows:

(a) for the virus induced expression of particular gene sequences e.g. desirable peptide products, proteins which will interfere with virus infection e.g. virus induced expression of commercially important peptides or cell-lethal proteins which might contain spread of the virus or other pathogens;

(b) for the virus induced expression of particular nucleic acid sequences with biological activity, from

primary transcripts which contain the inactive complementary strand of that nucleic acid e.g. virus induced production of the ribozyme targeted against RNA components of the virus genome (if it is an RNA virus). or its transcripts of its genetic information;

(c) for the production of either of the above in a transgenic eukaryote which contains the virus replicase genes linked to an inducible promoter, such that the induction of the replicase results in the subsequent production of the desired RNA strand in the cells.

It should also be appreciated that a negative sense RNA is not capable of translation, and hence heterologous gene products will only be produced following production of a positive transcript which occurs only when transcription is initiated from the sub-genomic promoter.

This invention will now be described with reference to the following non-limiting Figures and Examples which are exemplary but not restrictive upon the scope of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS:

FIGURE 1 Structure of gene construction (top) and transcription/replication path of RNA transcript (shown by arrows);

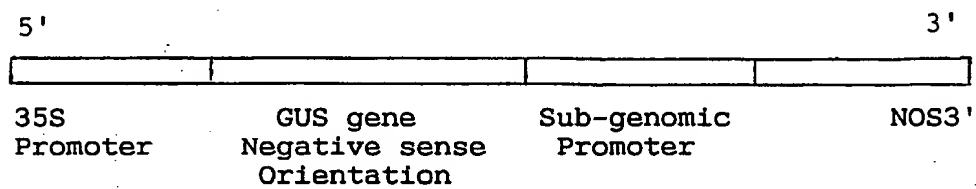
FIGURE 2 Relative GUS activity in protoplasts 48 hours after electroporation of the following constructs (a) infectious BYDV RNA alone (b) SGP-GUS gene construct alone (c) BYDV RNA + SGP-GUS gene construct. GUS activity is determined by standard fluorometric assay.

EXAMPLE

Experiments were undertaken to determine whether the viral associated replicase complex will (i) recognise the sub-genomic promoter represented on the primary negative sense transcript resulting in amplified levels of positive sense transcript for the heterologous gene or, alternatively, plus strand sequence containing novel

nucleic acid motifs, and (ii) whether subsequent translation products from the newly synthesized plus strand RNA sequence of heterologous gene can be detected.

A gene construction was prepared as follows: (also shown in Figure 1)



The 35S promoter refers to Cauliflower mosaic virus 35S promoter. The GUS (β-glucuronidase) gene negative sense orientation refers to the structural gene for the GUS reporter protein oriented such that transcription from the 35S promoter produces transcript of the negative sense sequence of this gene.

The sub-genomic promoter is a 121 nucleotide intergenic sequence upstream (5') of coat protein gene of barley yellow dwarf virus (BYDV) genome. This region is assumed to contain a sub-genomic promoter sequence which is specifically recognised by the BYDV replicase complex during virus replication, resulting in a sub-genomic mRNA for BYDV coat protein. Production of coat protein sub-genomic mRNA is assumed to only be initiated by internal initiation by the viral replicase complex from the sub-genomic promoter sequence as it is represented on the negative sense of the viral genome.

NOS 3' refers to nopaline synthase polyadenylation signals.

The 121 nucleotide BYDV coat protein sub-genomic promoter is fused to the GUS reporter gene so that transcription directed by the 35S promoter directs the synthesis of a negative sense GUS-sub-genomic RNA. Recognition of the sub-genomic promoter sequence represented on the negative sense GUS-sub-genomic promoter by the viral replicase complex results in production of positive sense mRNA for GUS.

The above gene construction was produced and propagated in bacterial plasmid vectors using standard recombinant DNA techniques.

One example of the experiment involved introduction into *Triticum monococcum* protoplasts of both the GUS-sub-genomic promoter gene construction as contained on the plasmid, along with infectious RNA of barley yellow dwarf virus. Two appropriate controls were also included: (i) The GUS-SGP plasmid construct introduced into the protoplasts alone, and (ii) the infectious BYDV RNA introduced into the protoplasts alone. Gus activity was assayed in the protoplast samples after an appropriate interval.

Gus activity results for different samples are shown in Figure 2.

Significant GUS activity was detected only when infectious BYDV RNA was co-introduced into the cells along with the SGP-GUS transcript. This is assumed to occur via recognition of the sub-genomic promoter by the virus replication system, so that positive sense mRNA is produced for the GUS gene, and thus is translated to produce GUS protein and subsequent GUS activity.

The above example demonstrates how activity of a particular gene (encoding β-glucuronidase) is amplified. It is clear from this example that any desired gene activity may be amplified utilizing one or more sub-genomic promoters.

CLAIMS:

1. A nucleic acid sequence characterised in that it comprises:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript.

2. A nucleic acid sequence according to claim 1 characterised in that said heterologous gene sequence is in an orientation which on transcription gives a negative sense primary RNA transcript.

3. A nucleic acid sequence according to claim 1 characterised in that said heterologous gene sequence is in an orientation which on transcription gives a positive sense primary RNA transcript.

4. A nucleic acid sequence according to claim 1 or 2 wherein said heterologous gene sequence encodes a desired polypeptide product.

5. A nucleic acid sequence according to claim 4, wherein said peptide product is an antibiotic, toxin, hormone, protease or viral protein.

6. A nucleic acid sequence according to claim 4, wherein said peptide product interfers with virus infection or modifies phenotype, sterility, salt

tolerance, virus susceptibility, drought tolerance, acidity, or colour of a plant.

7. A nucleic acid sequence according to claim 1 or claim 3, wherein said heterologous gene sequence encodes one or more endoribonucleases capable of site specific cleavage of target RNAs, or an RNA sequence which is substantially complementary in sequence to a cellular RNA or viral RNA selected for inactivation.

8. A nucleic acid sequence according to claim 1, which additionally comprises a gene encoding a selectable marker.

9. A nucleic acid sequence according to claim 8, wherein said selectable marker confers antibiotic resistance, herbicide resistance, colour change, or encodes a polypeptide which reacts with a substrate to produce a detectable signal.

10. A nucleic acid sequence according to claim 1 which additionally comprises nucleotide sequences which facilitate integration of said nucleic acid sequences into chromosomal DNA of eukaryotic or prokaryotic cells.

11. A nucleic acid sequence according to claim 10, wherein said nucleotide sequences comprise viral long terminal repeats (LTR's), or T-DNA of the Ti plasmid of Agrobacterium.

12. A nucleic acid sequence according to claim 1, characterised that the transcriptional promoter is a eukaryotic promoter.

13. A nucleic acid sequence according to claim 12, wherein said promoter is a plant specific promoter.

14. A nucleic acid sequence according to claim 12, wherein said plant specific promoter is a promoter of a plant virus.

15. A nucleic acid sequence according to claim 1, wherein said sub-genomic promoter is of plant or animal viral origin.

16. A nucleic acid sequence according to claim 15, wherein said sub-genomic promoter interacts with an RNA dependent RNA polymerase of a plant viral replicase complex.

17. A nucleic acid sequence according to claim 1 which comprises multiple sub-genomic promoter sequences.

18. A nucleic acid sequence according to claim 17, wherein each sub-genomic promoter sequence of the multiple sub-genomic promoter sequences is specific for a particular plant virus polymerase.

19. A nucleic acid sequence according to claim 1, wherein a complementary sequence of a sub-genomic promoter is inserted between the transcriptional promoter and the heterologous gene sequence, such that said secondary transcripts are transcribed from the sub-genomic promoter produced on transcription of the primary transcript to give a plurality of tertiary transcripts.

20. A transfer vector containing a nucleic acid sequence as claimed in any one of claims 1 to 19.

21. A transfer vector according to claim 20 which is a plasmid or phage transfer vector.

22. A eukaryotic cell transformed with a nucleic acid sequence according to any one of claims 1 to 19.

23. A eukaryotic cell according to claim 22, wherein said nucleic acid sequence is integrated into the genome of said eukaryotic cell or is present as an extra-chromosomal element.

24. A eukaryotic cell according to any of claims 22 to 23 wherein said cell is a plant or animal cell.

25. A transgenic plant containing a nucleic acid sequence according to any one of claims 1 to 19.

26. A transgenic plant according to claim 25 selected from wheat, barley, rye, sorghum, potato, rice, rape and apple.

27. A transgenic animal containing a nucleic acid sequence according to any of claims 1 to 19.

28. A process for transcriptional amplification, which process comprises:

(i) providing a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

(ii) reacting said nucleic acid sequence with a DNA dependent RNA polymerase such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript; and

(iii) reacting said primary transcript with an RNA dependent RNA polymerase to thereby effect transcription of said primary transcript from the sub-genomic promoter to give a plurality of amplified secondary transcripts.

29. A method for protecting a plant or animal cell

from viral infection, which method comprises introducing into one or more cells of said plant or animal a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript;

and wherein the primary transcript produced under control of said transcriptional promoter is transcribed from the sub-genomic promoter by an RNA polymerase to give a secondary transcript which encodes a polypeptide product, or which itself, inhibits viral replication or inactivates said virus.

30. A method for producing a heterologous gene product in a cell, which method comprises introducing into said cell a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter;

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

reacting said primary transcript produced on cellular transcription of said nucleic acid sequence with an RNA dependent RNA polymerase capable of transcription from said sub-genomic promoter to give a secondary transcript which itself may comprise said heterologous gene product, or which may be translated in the cell to give a heterologous gene product.

31. A method for inactivating an exog nous or endogenous cellular RNA, which method comprises introducging into a cell a nucleic acid sequence comprising:

(a) a transcriptional promoter;

(b) a heterologous gene sequence operably linked to said transcriptional promoter; and

(c) a sub-genomic promoter ligated to said heterologous gene sequence;

such that the heterologous gene sequence and sub-genomic promoter are transcribed under the action of said transcriptional promoter to give a primary transcript, and said primary transcript is capable of transcription under the action of said sub-genomic promoter to give a secondary transcript;

wherein the primary transcript produced under control of said transcriptional promoter is transcribed from the sub-genomic promoter by an RNA polymerase to give a secondary transcript, and wherein said secondary transcript or a translated product thereof inactivates said exogenous or endogenous cellular RNA.

32. A method according to claim 31 wherein said nucleic acid sequence is a nucleic acid sequence according to claim 19 and wherein said tertiary transcripts or translated products thereof inactivate said exogenous or endogenous cellular RNA.

33. A method according to claim 29, wherein transcription from said sub-genomic promoter is effected by an RNA polymerase of the infecting virus.

34. A method according to any one of claims 29 to 31 wherein transcription from said sub-genomic promoter is effected by an RNA polymerase produced within the cell.

35. A method according to claim 34, wherein the RNA polymerase is produced by a gene integrated into the host genome or carried by an extrachromosomal element.

36. A process according to claim 28, wherein said nucleic acid sequence comprises a nucleic acid sequence according to claim 19.

37. A method according to claim 29, wherein said nucleic acid sequence is a nucleic acid sequence according to claim 19 and wherein tertiary transcripts or translated products thereof produced on transcription from the secondary transcripts inhibit viral replication or inactivate said virus.

38. A method according to claim 30 wherein said nucleic acid sequence is a nucleic acid sequence according to claim 19 and wherein tertiary transcripts produced or translated products thereof produced on transcription from the secondary transcripts comprise said heterologous gene product.

39. A method according to claims 31 or 31 which is a method for the modification of cellular phenotype, sterility, salt tolerance, virus susceptibility, drought tolerance, acidity or colour of a plant.

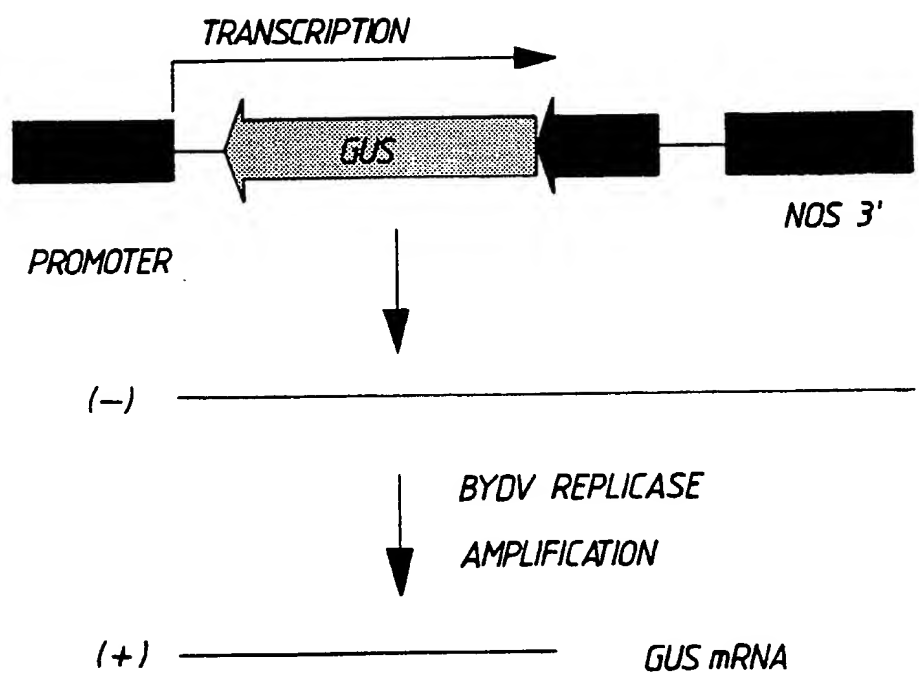

TRANSCRIPTION
GUS
PROMOTER
NOS 3'
(−)
BYDV REPLICASE
AMPLIFICATION
(+)
GUS mRNA

Fig.1.

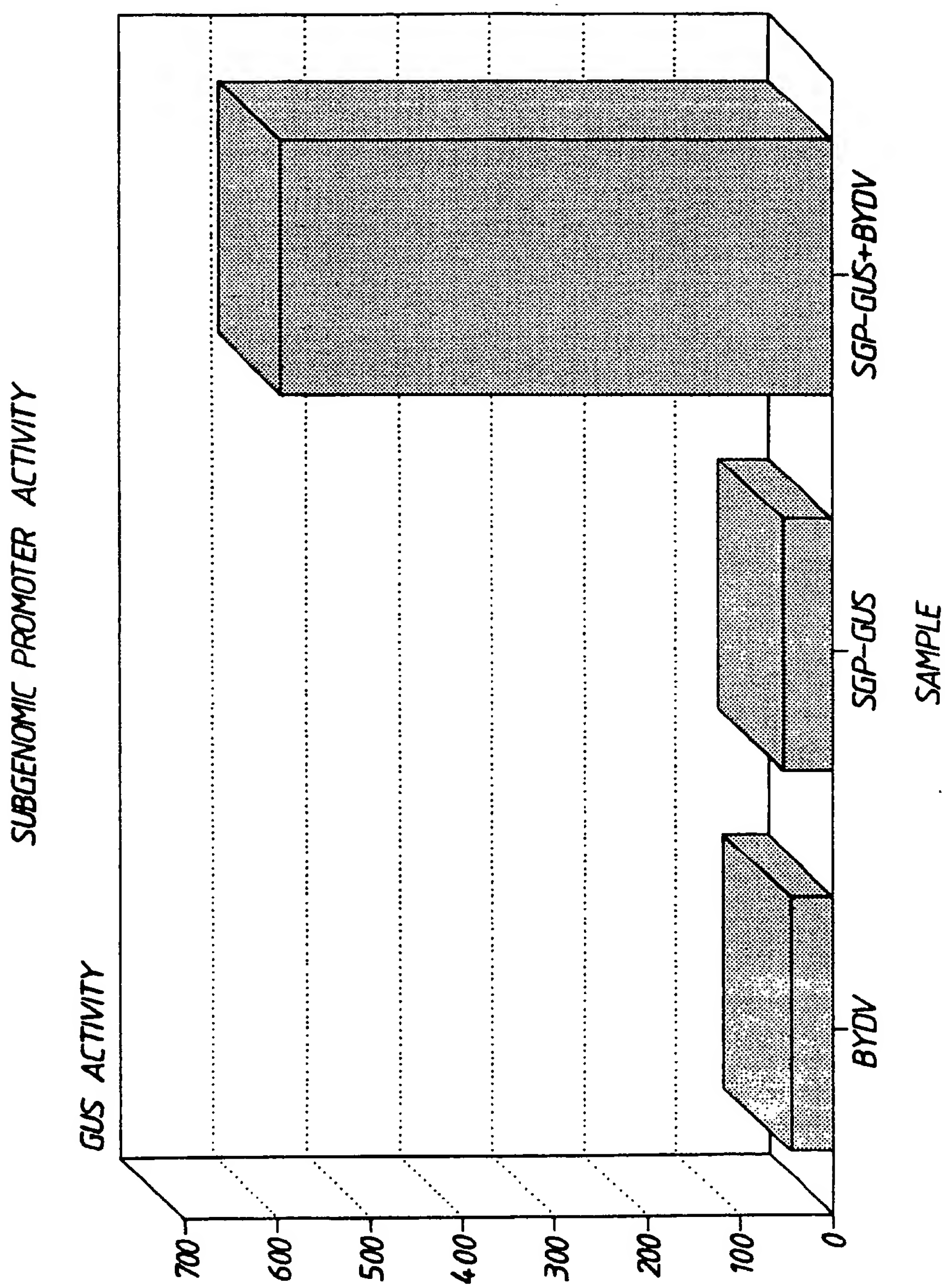

SUBGENOMIC PROMOTER ACTIVITY
GUS ACTIVITY
700
600
500
400
300
200
100
0
BYDV
SGP-GUS
SGP-GUS+BYDV
SAMPLE

Fig.2.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 91/00088

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.^{5} : C12N 15/83, C12Q 1/68, A01H 5/00, A01K 67/027

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC^5	DERWENT DATABASE : WPAT KEYWORDS : IC C12N 15/-; IC C12Q 1/68; promoter; transcript:; amplif:
Chem Abstr.	promoter; transcript:; amplif:; sub-genom:

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AUSTRALIAN SEARCH : IPC^5 C12N 15/82, C12N 15/83, C12Q 1/68
BIOT : KEYWORDS as above for WPAT and DWARF

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	AU,A, 21265/88 (SISKA DIAGNOSTICS) 16 March 1989 (16.03.89) see pages 16-23, the formulae in particular and the definitions of variable subsegment, primer and promoter; see page 26, line 31 - page 27, line 24 read in light of above pages.	(2-5,8,10-17,19, 28,36)
A	Frankham, R. (1988) Molecular Hypotheses for Position-Effect Variegation Anti-Sense Transcription and Promoter Occlusion, Journal of Theoretical Biology, Volume 135, No.1, pages 85-107; June, 1988.	
A	Coutts, R.H.A. and Buck, K.W. (1986) Studies on the Replication of Gemini Viruses and Construction of Gene Vectors, J. Cell. Biochem, Suppl. 10C, page 38; Conference Abstract.	

(continued)

* Special categories of cited documents: 10

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 June 1991 (27.06.91)	4 July 1991
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	Karen Ayers K. AYERS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	WO,A, 8703905 (GENETICS INSTITUTE, INC.) 2 July 1987 (02.07.87)	
A	EP,A2, 329822 (CANGENE, CORP.) 30 August 1989 (30.08.89)	
A	AU,B, 63924/84 (607122) (CETUS CORPORATION and GRACE ASC CORPORATION) 30 April 1987 (30.04.87)	

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest
[ ] The additional search fees were accompanied by applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL APPLICATION NO. PCT/AU 91/00088

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 21265/88	BR 8807097	DK 6444/89	EP 368906
	ES 2009286	FI 896077	HU 52823
	IL 86724	JP 2500565	NO 895090
	PT 87769	WO 8810315	ZA 8804350
WO 8703905	EP 277954	JP 63502719	US 4963481
EP 329822	WO 9102814 (7 March 1991)		JP 2005864
	ZA 8906476	AU 41858/89	
AU 63924/86	BR 8605043	EP 223399	

END OF ANNEX